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Short communication

1,4-Anthraquinone: A new useful pre-column reagent for the determination of *N*-acetylcysteine and captopril in pharmaceuticals by high performance liquid chromatography

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ABSTRACT

1,4-Anthraquinone (ANQ) is proposed as a novel pre-column reagent for high performance liquid chromatography (HPLC) determination of *N*-acetylcysteine (NAC) and captopril (CAP) in pharmaceutical formulations. The derivatization reactions were carried out at room temperature: NAC at pH 8 for 1 min, while CAP at pH 7.5 for 20 min. Both reactions reached completeness at a reagent to thiol molar ratio of about 2.5. The synthesised derivatives were characterized by ^{1}H NMR and IR. The chromatographic separations were performed on a C_{18} Phenomenex Synergi Fusion 4 μm (250 mm × 4.6 mm I.D.) *stainless* steel column with detection at λ = 300 nm. The mobile phase consisted of methanol/triethylammonium (TEA) phosphate buffer (pH 3; 0.05 mol/L) 75:25 (v/v) at a flow-rate of 0.4 mL/min for NAC and 88:12 (v/v), at a flow-rate of 0.6 mL/min for CAP. The validation parameters (linearity, sensitivity, accuracy, precision, specificity and stability) were highly satisfactory. Linear response was observed (determination coefficient ≥0.9996). Detection limits were about 8 and 18 ng/mL for NAC and CAP, respectively. Intra-day precision (relative standard deviation, R.S.D.) was ≤1.58%, for thiol to internal standard (IS) peak area ratio and ≤0.33%, for thiol and IS retention times (t_R), without significant differences between intra- and inter-day data. Thiol recovery studies were satisfactory (99.50%) with R.S.D. ≤0.56%. The results highlight the high sensitivity of the method and the remarkable reactivity and selectivity of the reagent towards the thiol function. The developed method is suitable for the quality control of both thiols in commercial products. The method can be applied in any analytical laboratory not requiring a sophisticated instrumentation.

1. Introduction

N-Acetylcysteine (NAC) is an intermediate in the conversion of cysteine to glutathione. Antioxidants such as NAC have been used as tools for investigating the role of reactive oxygen species (ROS) in numerous biological and pathological processes. NAC is used as a mucolytic agent and as an antidote in case of poisoning by acetominofen [1–5]. Captopril (CAP, 1-[3-mercapto-2-(*S*)-methyl-1-oxopropyl]-(*S*)-proline) is a potent, competitive inhibitor of angiotensin-converting enzyme (ACE). CAP is widely used in the treatment of essential hypertension and some types of congestive heart failure. As an agent, it has been proposed to complex cysteine in the treatment of cystinuria [1,2,6,7]. Different LC methods have been employed to determine thiols including primarily reversed phase liquid chromatography (RP-LC), ion pair liquid chromatography (IP-LC), and ion exchange chromatography [2,5,6,8]. Chemical derivatization plays a predominant role, because it allows the modification of substances with low UV absorption or compounds, which do not have a chromophore or fluorophore group into highly sensitive products [9]. Derivatization can improve the chromatographic retention of polar compounds and resolution of closely eluted compounds, because the derivatives are typically more hydrophobic than the underivatized analyte. The pre-column derivatization is very significant for thiol analysis, because they might be decomposed during the separation in the analytical column [2]. There are numerous thiol-reactive reagents, commercially available or made in laboratory that permit UV-absorbance or fluorescence detection [2,5,6,9–29]. Unfortunately, most reagents can involve different drawbacks such as increased reaction times and steps, as well as increased cost, complexity of the analysis due to reagent excess, and side reaction products [2,6,9–23]. Currently, no method reports the use of 1,4-anthraquinone (ANQ) as an analytical

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reagent. ANQ is an anticancer drug [30] and is a compound commercially available that belongs to the category of the activated double bond compounds. ANQ molecular structure is similar to that of NPQ and MD [28,29], but has a higher UV-absorptivity for the presence of an aromatic ring condensed to the naphthalene nucleus. The main target of this work is to propose ANQ as a new derivatization reagent for its significant reactivity and selectivity towards the thiol group and potentially more sensitive in comparison with NPQ and MD. The different parameters affecting the derivatization reaction were experimentally studied. The thiol-derivatives were synthesised on a preparative scale and characterized by ^{1}H NMR and IR techniques.The validated method was applied to the quali-quantitative analysis of NAC and CAP in commercial dosage forms.

2. Experimental

2.1. Materials and solutions

CAP, sodium 2-mercaptoethanesulfonate, (MeSNa), reduced glutathione (GSH), thioglycolic acid, papaverine as internal standard (IS), methanol Chromasolv®, acetonitrile, chloroform and acetic acid were obtained from Sigma-Aldrich (Milan, Italy). NAC, D,L-homocysteine (Hcy), *N*-(2-mercaptopropionyl)glycine (thiola), boric acid were obtained from Fluka (Milan, Italy). 1,4-Antraquinone (ANQ) reagent was purchased from Alfa Aesar (Karlsruhe, Germany). Triethylamine was purchased from Carlo Erba (Milan, Italy). TLC was performed on Bakerflex plates (Silica gel IB2-F) (Phillipsburg N.J., USA). Purified water by a Milli-RX apparatus (Millipore, Milford, MA, USA) using 0.22 μm filters was employed for the preparation of all solutions, buffers and mobile phase. All formulations (effervescent tablets, tablets and medicines in packets) were purchased from a local pharmacy. All solutions were prepared freshly and stored at 2–8° C during the day. IS stock solution (about 6.5 mM) was prepared in methanol. NAC and CAP standard solutions (concentration as calibration ranges) were prepared in presence of IS (about 0.44 mg/mL) using a mixture of methanol/borate buffer (0.1 M) at pH 8 for NAC and pH 7.5 for CAP in the ratio 80:20 (v/v). ANQ standard solutions (3.3 mM) were prepared in methanol. The borate buffer solutions (pH 8 and pH 7.5; 0.1 M) were prepared by dissolving boric acid in water and adjusting to the desired pH with 2N sodium hydroxide according to standard methods. TEA phosphate buffer solution (pH 3; 0.05 M) was prepared by adding orthophosphoric acid to an aqueous triethylamine solution up to the desired pH value.

2.2. Equipment

The liquid chromatograph consisted of a PU-1580 pump equipped with the LG-1580-02 ternary gradient unit and a diode-array detector (DAD) model MD-910 (Jasco Corporation, Tokyo, Japan). The data were collected on a PC equipped with the integration program Borwin-PDA. The solvents were degassed on line with a degasser model DG 2080-53 (Jasco Corporation). Manual injections were carried out using a Rheodyne model 7725i injector with 20 μL sample loop. A column inlet filter (0.5 μm × 3 mm i.d.) model 7335 Rheodyne was used. Infrared spectra (IR) were measured on a Jasco FT-IR instrument (Jasco Corporation, Tokyo, Japan). The ^{1}H NMR spectra were recorded in $(CD_3)_2SO$ on a Varian MR 400 MHz (ATB PFG probe); the chemical shift (referenced to solvent signal) is expressed in δ (ppm) and J in Hz. ReactiTherm/Heating/Stirring module (Pierce, Rockford, IL, USA) was used for the derivatization reaction. Sonarex Super RK 102 (35 KMZ) Bandelin (Berlin, Germany) equipment with thermostatically controlled heating (30–80 °C) was used for ultrasonication.

2.3. Synthesis of NAC and CAP derivatives of ANQ

0.36 mmol of the appropriate thiol (NAC or CAP) dissolved in 20 mL of water were added to about 75 mg ANQ (0.36 mmol) in 10 mL of methanol. The reaction mixture was stirred at room temperature (r.t.) for 5 min, acidified with 2N hydrochloric acid (about pH 1–2) and then extracted with chloroform (3 × 10 mL). The combined chloroform extracts were washed with sodium bicarbonate 10% (3 × 10 mL). The aqueous solution was acidified to obtain a precipitate, which was collected by filtration. The solid was found to be pure by TLC (mobile phase: chloroform/methanol/acetic acid 9:1:0.5 (v/v/v); UV detection at λ = 254 and 366 nm). Experimental data of the derivatives are reported below (ar aromatic).

2.3.1. NAC-ANQ derivative: (N-*acetyl*-S-*(1,4-dioxo-1,4-dihydrodroanthracen-2-yl)*-D-*cysteine)*

$C_{19}H_{15}NO_5S$; MW 369.39; mp = 165–170 °C with dec., uncorrected value. IR (in KBr, cm^{-1}): 3450, 1666, 1279, 1030, 751. ^{1}H NMR (DMSO-d_6) δ: 1.87 (3H, s, CH_3), 3.23 (1H, dd, J = 13.5, J = 8.4, CH_2), 3.40 (1H, dd, J = 13.5, J = 4.8, CH_2), 4.57 (1H, m, CH NAC), 6.96 (1H, s, ar), 7.79 (2H, m, ar), 8.30 (2H, m,ar), 8.45 (1H, d, J = 7.2, NH), 8.64 (1H, s, ar), 8.70 (1H, s, ar), 13.13 (1H, s broad, COOH). After D_2O addition, owing to proton exchange of NH group, a simplification of the cysteinic CH signal was observed. The ^{1}H NMR spectra changes as follows: 1.86 (3H, s, CH_3), 3.21 (1H, dd, J = 13.5, J = 8.4, CH_2), 3.39 (1H, dd, J = 13.5, J = 4.8, CH_2), 4.52 (1H, dd, J = 8.4, J = 4.8, CH NAC), 6.91 (1H, s, ar), 7.78 (2H, m, ar), 8.26 (2H, m, ar), 8.64 (1H, s, ar), 8.70 (1H, s, ar).

2.3.2. CAP-ANQ derivative: (((R)-*3-((1,4-dioxo-1,4-dihydroanthracen-2-yl)thio)-2-methylpropanoyl)*-L-*proline)*

$C_{23}H_{21}NO_5S$; MW 423.48; mp = 125–130 °C, uncorrected value. IR (in KBr, cm^{-1}): 3450, 1616, 1274, 1191, 756. ^{1}H NMR (DMSO-d_6) δ: 1.21 (3H, d, J = 6.4, CH_3); 1.87 (3H, m), 2.15 (1H, m), 3.03 (2H, m), 3.16 (1H, m), 3.57 (2H, m), 4.26 (1H, dd, J = 8.6, J = 3.8, CH), 6.92 (1H, s, ar); 7.78 (2H, m, ar), 8.29 (2H, m, ar), 8.60 (1H, s, ar), 8.67 (1H, s, ar), 12.35 (1H, s broad, COOH).

2.4. Derivatization procedure

A 200 μL aliquot of limpid thiol solution (standard or sample after filtration) in presence of IS was treated with 100 μL of ANQ. The reaction was carried out at r.t. for 1 and 20 min for NAC and CAP, respectively, under magnetic stirring in a micro-reaction vessel (3.0 mL). Then, 200 μL of mobile phase were added. Finally, a 20 μL aliquot of the obtained solution was injected in triplicate into the chromatographic system.

2.5. Chromatographic conditions

HPLC separations of the derivatized thiols were performed at r.t. on a C_{18} Phenomenex Synergi Fusion 4 μm, (250 mm × 4.6 mm I.D.) stainless steel column with a guard column packed with the same stationary phase. A mobile phase consisting of methanol (eluent A) and TEA phosphate buffer pH 3 (eluent B) 75:25 (v/v) was used at a flow-rate of 0.4 mL/min for NAC routine analysis, and eluent A to eluent B was 88:12 (v/v) at a flow-rate of 0.6 mL/min for CAP. The chromatographic separation of all examined thiols was carried out with the same mobile phase under the following gradient conditions: from 0 to 36 min, 42% of B; from 36 to 40 min 22% of B; from 40 to 50 min 22% of B; from 50 to 60 min, 42% of B. The flow-rate was 0.4 mL/min. UV-DAD was used setting the wavelength at λ = 300 nm.